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Human alpha 1-antitrypsin therapy induces fatal anaphylaxis in non-obese diabetic mice

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Abbreviations: AAT, alpha-1 antitrypsin; NOD mice, nonobese diabetic mice.

Summary

Previous studies have shown that human alpha-1 antitrypsin (hAAT) gene delivery prevents type 1 diabetes (T1D) in non-obese diabetic (NOD) mice. Furthermore, hAAT protein administration prolongs acceptance of islet allografts. Therefore, we evaluated the use of purified hAAT protein therapy to prevent T1D in NOD mice. Female NOD, non-obese resistant (NOR), Balb/c and C57BL/6 mice were injected intraperitoneally with vehicle alone or vehicle containing hAAT, human albumin or mouse albumin (or mg/injection/mouse; 2×/week). Preparations of clinical-grade hAAT included API®, Aralast®, Prolastin® and Zemaira®. Surprisingly, hAAT administration was associated with a high rate of fatal anaphylaxis. In studies seeking T1D prevention at 4 weeks of age, 100% mice died after six injections of hAAT. When administrated at 8–10 weeks of age, most (80–100%) NOD mice died following the fourth injection of hAAT, while 0% of Balb/c and C57BL/6 mice and 10% of NOR mice died. Interestingly, repeated injections of human albumin, but not mouse albumin, also induced sudden death in NOD mice. Antibodies to hAAT were induced 2–3 weeks after hAAT administration and death was prevented by treatment with anti-platelet-activating factor along with anti-histamine. In studies of disease reversal in NOD mice, using the four pharmaceutical grade formulations of hAAT, anaphylactic deaths were observed with all hAAT preparations. The propensity for fatal anaphylaxis following antigenic administration appears to be NOD- but not hAAT-specific. The susceptibility of NOD mice to hypersensitivity provides a significant limitation for testing of hAAT. Development of strategies to avoid this unwanted response is required to use this promising therapeutic agent for T1D.

Keywords: alpha 1 antitrypsin, anaphylaxis, NOD mice, type 1 diabetes

Introduction

Non-obese diabetic (NOD) mice have been utilized widely as a prototypic animal model for studies of type 1 diabetes (T1D), given their spontaneous development of hyperglycaemia and a disease whose pathogenic basis appears autoimmune in nature [1,2]. Indeed, NOD mice have proved themselves to be a highly valuable model for T1D studies related to genetics, immunology and islet pathology [3]. These immunogenetic similarities have also led to their use in the testing of therapeutic agents that may be beneficial for preventing or reversing T1D in humans [4]. To this capability, we and others have shown previously the efficacy of human alpha-1 antitrypsin (hAAT) to provide a pronounced therapeutic benefit in settings potentially meaningful to those with T1D. Specifically, we demonstrated that early (i.e. 4–5 weeks) gene delivery of hAAT to NOD mice prevents T1D development [5,6]. In addition, Lewis *et al.* have shown that administration of clinical-grade hAAT prolongs islet allograft survival in C57BL/6 mice[7].

AAT is a serine proteinase inhibitor with anti-inflammatory properties that has a role in innate immunity and in protection from tissue damage. Notably, AAT inhibits neutrophil elastase, proteinase 3, cathepsin G, trypsin and many other proteinases. In the context of T1D development in NOD mice, the studies of AAT gene delivery suggested that the treatment both reduces the levels of insulin autoantibodies and attenuates the development of insulitis [5,6].

Recent studies (both *in vitro* and *in vivo*) have also demonstrated that hAAT inhibits caspase 3 activity and protects islet cells from apoptosis [8–10]. Therefore, in an ongoing effort to transition this promising form of therapy from mice to humans, we initiated a series of studies addressing the question of whether administration of clinical-grade hAAT would be effective for both the prevention and reversal of T1D in NOD mice.

Materials and methods

Mice

Female NOD/LtJ, non-obese resistant (NOR)/LtJ, Balb/cJ and C57BL/6J mice were purchased from the Jackson Laboratory (Bar Harbor, ME, USA). Mice were housed in specific pathogen-free facilities at the University of Florida (Gainesville, FL, USA) and at the University of Miami (Miami, FL, USA). The Institutional Animal Care and Use Committees at both Universities approved all animal manipulations.

Therapeutic regimens

A variety of clinical-grade hAAT preparations were obtained, including Prolastin® (Bayer, Elkhart, IN, USA), Aralast® (Alpha Therapeutic Corp., Los Angeles, CA, USA), Zemaira® (Aventis, Kankakee, IL, USA) and API® (Kamada, Beit Kama, Israel). A clinical grade of human albumin, Albuminar® (ZLB, Behring, Kankakee, IL, USA) as well as mouse albumin (Calbiochem, Gibbstown, NJ, USA) were used as controls. For all studies described herein, mice were injected intraperitoneally (i.p.) at a dose of 1 or 2 mg/mouse/injection, with an injection schedule of two injections per week. Blood glucose levels were monitored twice per week. Diabetes was defined by two consecutive (>24 h apart) non-fasting blood glucose levels >240 mg/dl. In studies addressing the ability of hAAT to reverse T1D, newly diagnosed mice received insulin therapy along with the different formulations of vehicle [phosphate-buffered saline (PBS)], albumin or hAAT. Insulin implants (Linshin Canada Inc., Toronto, Canada; one or two pellets/mouse) were placed subcutaneously under the mid-dorsal skin to maintain metabolic control necessary to sustain viability for 3–4 weeks. In the transplant experiments, islets (syngeneic: NOD-Scid → NOD, C57BL/6 → C57BL/6; allogeneic: C57BL/6 → NOD, DBA/2 → C57BL/6) were implanted under the kidney capsule of spontaneously diabetic NOD mice or chemically diabetic C57BL/6 mice [streptozotocin 200 mg/kg intravenously (i.v.)], as reported previously [11]. Recipients were treated i.p. with hAAT on days −1, 0, 3, 6 and 9 (for NOD recipients) and every 3 days thereafter (in C57BL/6 recipients only). Non-fasting glycaemia was monitored on whole blood from tail vein samples using portable glucometers (OneTouch Ultra; Lifescan Inc., Milpitas, CA, USA).

Mechanistic studies

Enzyme-linked immunosorbent assays (ELISAs) for hAAT levels were performed as described previously [6]. Antibody levels of anti-AAT-specific immunoglobulin (Ig)E or Ig(G, A, M) were detected by ELISA. Briefly, 96-well plates (Immulon 4; Dynex Technologies, Chantilly, VA, USA) were coated with purified hAAT (1 μg/well; Athens Research & Technology, Inc., Athens, GA, USA) in Voller's buffer overnight at 4°C. After 1 h of blocking with 3% bovine serum albumin, 50–100-fold diluted mouse serum samples were added to the plate, followed by incubation at 37°C for 1 h. Horseradish peroxidase (HRP)-conjugated goat anti-mouse IgE antibody (1:1500 dilution; Immunology Consultants Laboratory, Newberg, OR, USA) or HRP-conjugated goat anti-mouse Ig(G, A, M) (1:1500 dilution, Sigma, St Louis, MO, USA) was added and incubated at 37°C for 1 h. The plate was washed with PBS-Tween 20 between reactions. After reaction with the substrate o-phenylenediamine (Sigma), plates were read at 490 nm on an MRX microplate reader (Dynex Technologies, Chantilly, VA, USA). For studies of anaphylaxis, NOD mice were injected i.p. with 200 μg in 100 μl sterile saline of the anti-histamine triprolidine (Sigma) 45 min, and injected i.v. with 66 μg in 100 μl sterile saline at neutral pH of the anti-platelet-activating factor (PAF) CV-3988 (Wako Chemicals, Richmond, VA, USA), 5 min prior to AAT injection, in accordance with other previously published work [12].

Statistics

Significance was determined by Student's *t*-test (GraphPad Prism, San Diego, CA, USA), with $P < 5$ deemed significant.

Results

Multiple administrations of human AAT induce fatal anaphylaxis in NOD mice prior to the onset of diabetes

In order to test the efficacy of clinical-grade hAAT (Prolastin®) to attenuate the development of T1D, 26 female NOD mice were injected i.p. beginning at 4 weeks of age. Surprisingly, beginning with the fourth injection and continuing throughout subsequent injections, animals died within 30 min of injection (Fig. 1a). These mice displayed classic signs associated with anaphylaxis and shock, including difficulty in breathing and collapse. The mortality rate increased with the number of injections (Fig. 1a). In similar studies designed to test whether hAAT intervention would be effective later in the natural history of T1D, 10-week-old NOD mice were injected with hAAT (Prolastin®; $n = 10$). In these experiments, 90% of mice died within 30 min following the fourth injection (Fig. 1b).

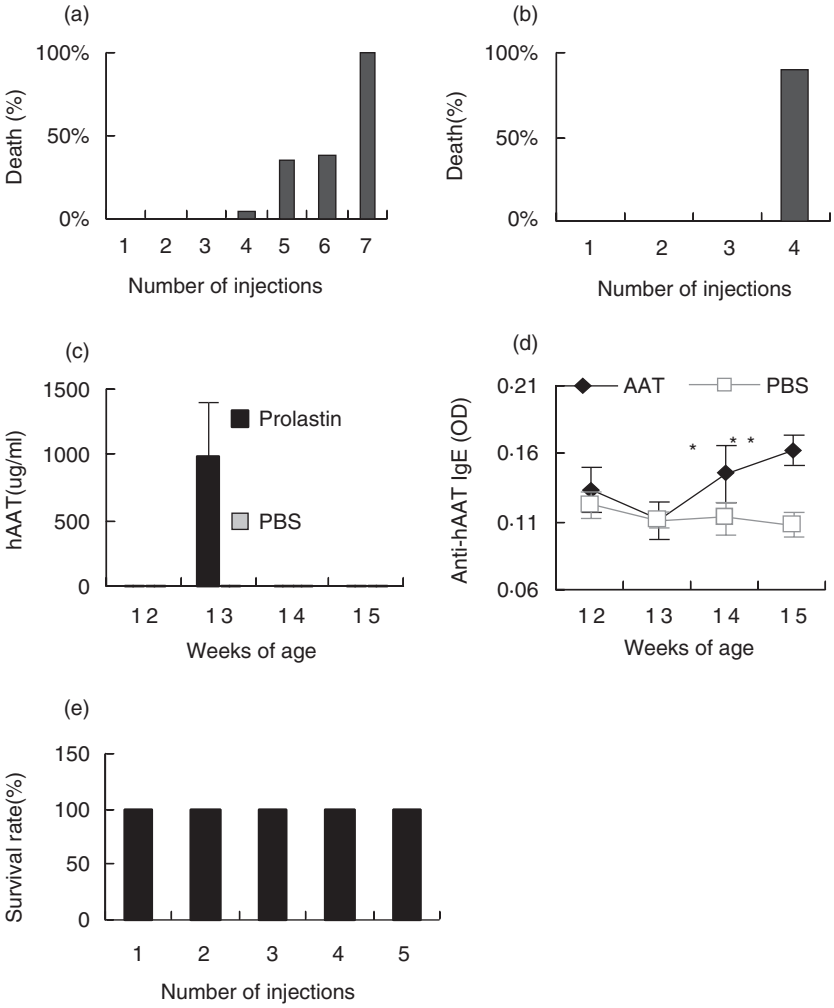

Fig. 1. Fatal anaphylaxis in non-obese diabetic (NOD) mice receiving human alpha-1 antitrypsin (hAAT) (Prolastin®, 1 mg/mouse/injection, two injections/week). (a) Five-week-old female NOD mice ($n = 26$) were injected intraperitoneally (i.p.); (b) 10-week-old mice ($n = 10$) were injected i.p. Each bar represents the death rate of each injection. (c, d) Effect of two injections of hAAT ($n = 6$) or phosphate-buffered saline ($n = 5$) at 12 weeks of age. hAAT levels were monitored by enzyme-linked immunosorbent assay (ELISA) (c); serum anti-hAAT immunoglobulin E (IgE) levels detected by ELISA (d) Each line represents the average optical density at 50× dilution. $^*P < 5$; $^{**}P < 1$. (e) Treatment of anti-platelet-activating factor and anti-histamine prior to the fourth and fifth injections of hAAT prevented anaphylaxis. Each bar represents the survival rate after hAAT injection ($n = 4$).

Administration of human AAT increases anti-AAT IgE levels

Given these observations, we sought to affirm the concept of type 1 hypersensitivity induction by monitoring both serum hAAT- as well as anti-AAT-specific IgE levels in NOD mice provided with repeated injections of hAAT (Prolastin®). In addition, we also questioned whether appreciable levels of hAAT could be obtained, and fatal anaphylaxis avoided, with a reduction to two injections. With this revised schedule involving two injections of NOD mice, no fatal anaphylaxis was observed (0/6; 0%). At baseline, serum levels of hAAT levels were, as expected, absent, but rose within 1 week of the two injections (Fig. 1c). Consistent with the estimated half-life of AAT *in vivo*, serum levels of hAAT also declined to baseline levels 1 week following cessation of the injections (Fig. 1c). However, in support of an IgE-mediated anaphylaxis reaction, serum anti-hAAT IgE levels were detectable 2 weeks following the initial injection and remained elevated (Fig. 1d). Furthermore, all NOD mice ($n = 4$, 12 weeks old) pretreated with anti-PAF and anti-histamine prior to the fourth as well as the fifth injection, survived following subsequent hAAT administration (Fig. 1e).

Anaphylaxis is NOD-specific but not hAAT-specific

In order to address the specificity of the fatal anaphylaxis, we performed a series of experiments. First, we injected NOD, NOR or Balb/c mice (8 weeks of age) with hAAT (Prolastin® or Aralast®), human albumin (Albuminar®) or mouse albumin (2 mg/mouse, two injections per week). As shown in Fig. 2a, hAAT induced fatal anaphylaxis in NOD mice (80–100%; based on multiple experiments using two forms of hAAT), but not in Balb/c mice (0%). Although one of 10 NOR mice died after the fourth injection, the remaining mice survived up to 10 injections (end of the experiment). In separate but similar studies, chronic hAAT treatment did not induce anaphylaxis in C57BL/6 mice (Table 1), indicating that the anaphylaxis is NOD-specific. Interestingly, human but not mouse albumin also induced anaphylaxis in NOD mice (Fig. 2a and Table 1). Anti-hAAT IgE and Ig(G, A and M) levels were elevated in all hAAT-injected groups but not in the mouse albumin-injected group (Fig. 2b,c).

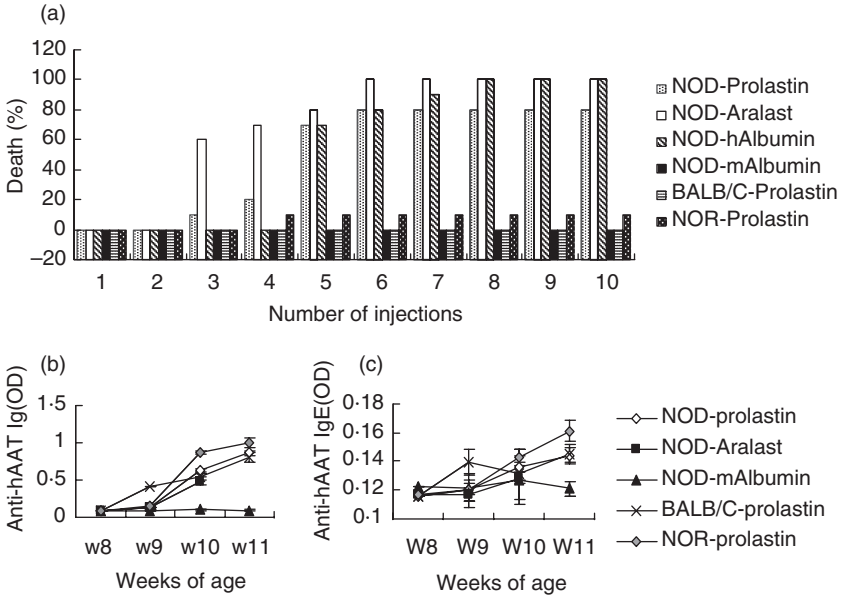

Fig. 2. Anaphylaxis is non-obese diabetic (NOD)-specific, but not human alpha-1 antitrypsin (hAAT)-specific. (a) Mortality rates in NOD, non-obese resistant and Balb/c mice (8 weeks of age) injected with hAAT (Prolastin® or Aralast®), human albumin (Albuminar®) or mouse albumin as indicated ($n = 10$, 2 mg/mouse, two injections per week); (b) anti-hAAT immunoglobulin G (IgG) levels. Each line represents the average optical density (OD) at 100× dilution; (c) anti-hAAT IgE levels. Each line represents the average OD at 50× dilution.

As one of our goals was to test the feasibility of hAAT to reverse T1D, we implemented two additional experimental modifications for the purpose of addressing the aforementioned limitation of anaphylaxis. First, to address the notion that the toxicity may be preparation-specific, we expanded testing to four preparations of clinical-grade hAAT. Secondly, we attempted to identify the number of administrations that could be provided without induction of anaphylaxis.

As shown in Table 1, cases of fatal anaphylaxis were observed with every hAAT preparation, as well as in mice receiving human albumin, following their fourth to sixth injection. To avoid further deaths, we stopped the injections. Of the remaining animals (i.e. those that survived treatment), no significant reversal of T1D was observed (Fig. 3). In transplanted NOD mice, fatal anaphylaxis was also observed following the fifth hAAT injection (i.e. day 9 post-

Table 1. Summary of several experiments (deaths/total number of injected mice).

		Injections					
Animals	Reagents	1	2	3	4	5	6
Non-diabetic†	Prolastin®	0/5	0/5	**1/5**	**4/4**		
NOD mice	Albuminar®	0/5	0/5	0/5	**4/5**	0/1	
	PBS	0/5	0/5	0/5	0/5	0/5	
Diabetic‡	API®	0/7	0/7	0/7	**1/7**	**2/5**	0/2
NOD mice	Aralast®	0/6	0/6	0/6	**1/6**	**2/3**	NA
	Prolastin®	0/5	0/5	0/5	0/5	**1/4**	NA
	Zemaira®	0/6	0/6	0/6	0/6	0/3	**1/3**
	Albuminar®	0/5	0/5	0/5	**1/5**	0/2	0/1
	PBS	0/4	0/4	0/4	0/1	0/1	NA
Transplanted diabetic	Aralast®	0/5	0/5	0/5	0/5	**2/5**	NA
NOD mice§	Prolastin®	0/20	0/20	0/20	0/20	**4/16**	NA
Transplanted diabetic	Aralast®	0/7	0/7	0/7	0/7	0/7	0/7
C57BL/6 mice¶	Prolastin®	0/23	0/23	0/23	0/23	0/23	0/23

Shown in bold type are animals in which deaths were observed. †Comparison of human alpha-1 antitrypsin (hAAT) (Prolastin®) with human albumin (Albuminar®); 18-week-old non-diabetic non-obese diabetic (NOD) mice were injected with Prolastin®, Albuminar® (2 mg/mouse/injection, two injections per week) or phosphate-buffered saline (PBS). ‡Comparison of different preparations of hAAT. Newly diagnosed NOD mice receive insulin treatment and hAAT, albumin (1 mg/ml, two injections per week) or PBS. §Effects of different preparations of hAAT (Aralast® and Prolastin® given at 1 or 2 mg/injection/mouse, given on days –1, 0 and every 3 days after transplantation) in spontaneously diabetic NOD mice receiving islet transplants. ¶Effects of different preparations of hAAT (Aralast® and Prolastin® given at 1 or 2 mg/injection/mouse, given on days –1, 0 and every 3 days after transplantation) in chemically induced diabetic C57BL/6 mice receiving islet transplantation. No deaths were observed in this strain under hAAT treatment even when up to 25 injections were given.

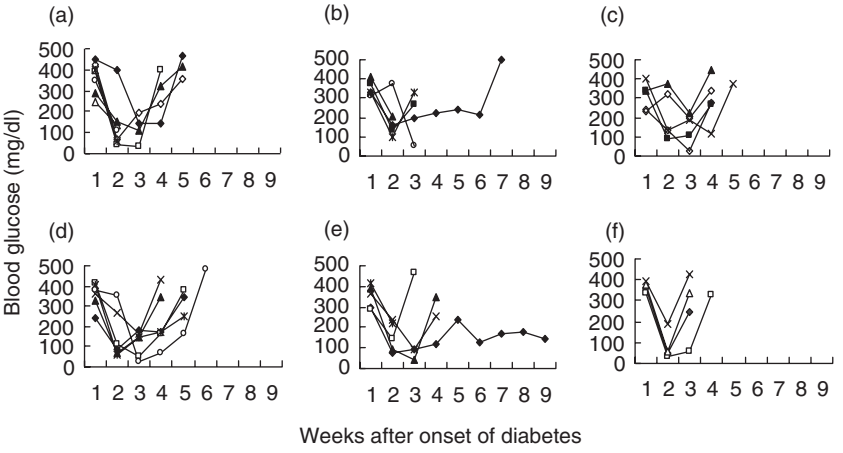

Fig. 3. Blood glucose levels in alpha-1 antitrypsin (hAAT)-treated diabetic mice. New-onset diabetic mice were treated with insulin and hAAT (1 mg/mouse, two injections per week) from different manufacturers. (a) API® from Kamada; (b) Aralast® from Alpha Therapeutic Corp.; (c) Prolastin® from Bayer; (d) Zemaira® from Aventis; (e) Albuminar® from ZLB Behring; (f) saline as a control.

transplant), while in C57BL/6 mice, no deaths were observed despite chronic hAAT treatment involving up to 25 injections (Table 1).

Discussion

As part of their susceptibility to T1D, a variety of immune system-related abnormalities [e.g. C5 deficiency, antigen-presenting cell (APC) dysfunction, interleukin (IL)-2 production, etc.] have been ascribed as being fundamental to the development of disease in NOD mice [1,2]. These immunological shortcomings result in an animal model that is not only susceptible to T1D, but in addition one that displays a variety of additional disease-related sequelae including thyroiditis and sialitis, as well as an enhanced proneness to the induction of experimental autoimmune encephalitis (EAE) [13–15]. In addition to enhanced susceptibility for autoimmune disease, NOD mice also display elevated immune responsiveness against foreign antigens, such as viral proteins, beyond those observed in many other inbred strains of mice [16]. Similarly, enhanced immune responsiveness in NOD mice has also been observed in our gene therapy studies where rAAV2-hAAT gene delivery induced high levels of anti-hAAT IgG in NOD mice, but not in C57BL/6 mice [5,16,17]. We observed high mortality following repeated hAAT administration in NOD mice. Furthermore, administration of anti-PAF and anti-histamines completely prevented their death. One could speculate that NOD mice may be more sensitive to histamine or degranulation of mast cells because, interestingly, all mice developed anti-hAAT IgE, including NOR, with 90% of survival suggesting that their presence alone was not sufficient to cause death.

Other studies have suggested that repeated injections of peptides may lead to fatal anaphylaxis in NOD mice [12,18,19]. Deleterious immune responses varied depending on the antigens and the route of administration. Interestingly, Liu *et al.* noted that anaphylaxis could be prevented by lowering the dose or altering the isoelectric point of the peptide to neutral pH [12,19,20].

A variety of protein-, peptide- or antibody-based therapies have been developed for the purpose of ameliorating T1D [21]. Of question is whether the application of these agents in NOD mice, when utilizing approaches involving repeated administration, will require additional considerations in order to overcome limitations of anaphylaxis susceptibility. Our studies revealed that repeated i.p. hAAT injections induced anaphylaxis in NOD mice of different ages and different disease stages within 2–3 weeks after the first dose. Purified human AAT protein has long been used for the treatment of AAT deficiency; a monogenic genetic defect leading to emphysema and liver disease [22,23]. As neither adverse events nor clinically relevant neutralizing antibodies against exogenously delivered hAAT have been observed routinely in AAT-deficient patients receiving clinical-grade AAT derived from human plasma, hAAT has long been considered a remarkably safe drug [24,25]. Furthermore, we have administered multiple doses of clinical-grade hAAT (Prolastin or Aralast) to hundreds of C57BL/6 and DBA/1 mice, without notation of significant adverse effects [7,8]. In the present studies of NOD mice, we observed fatal anaphylaxis against both hAAT as well as human albumin. It is possible that species difference between mouse and human proteins might contribute to fatal anaphylaxis in NOD mice. How albumin actually leads to anaphylaxis is the subject of ongoing studies.

A number of points are noteworthy with respect to these observations. First, the fatal anaphylaxis was not hAAT-specific, as similar adverse events were observed with human albumin. Secondly, in contrast to these results with hAAT protein therapy, our previous studies using recombinant adeno-associated virus delivered hAAT into NOD mice and other animal models did not lead to any fatal anaphylaxis [5,6,17,26]. Furthermore, this form of adverse event does, in fact, appear to be NOD-specific because administration of hAAT to other inbred strains of mice has not been associated with adverse events, including fatal anaphylaxis. Of particular note in this regard is the low percentage (10%) of fatal anaphylaxis in NOR mice, which are genetically very similar

to NOD, sharing the same human leukocyte antigen susceptibility alleles and other immune response abnormalities. This might indicate the need for ongoing autoimmune processes. However, our results that injections of hAAT at 5, 8, 10 and 12 weeks and after onset of T1D led to a similar degree of anaphylaxis suggest that the anaphylactic effect of hAAT in NOD mice is not related to disease progression. It will be of importance to ascertain whether this heightened sensitivity is occurring in other models for T1D, such as the rat insulin promoter–lymphocytic choriomeningitis virus (RIP-LCMV) model, or other autoimmune diseases (i.e. EAE, experimental autoimmune uveitits and collagen-induced arthritis models). Finally, in islet cell transplantation recipients, NOD mice receiving repeated administration of Prolastin® or Aralast® died shortly after their fifth injection, similar to what was observed in the diabetes prevention trials in NOD mice. In contrast, no deaths or signs of disease were observed in transplanted C57BL/6 mice, despite chronic administration of hAAT (up to 25 injections)[27].

Taken collectively, our concerns are that therapies of potential significance to prevent or reverse human T1D may be masked by the type 1 hypersensitivity reactions observed in NOD mice. While we do not believe that these findings should preclude testing of agents in human T1D for such purposes (indeed, to our knowledge, no such reactions have been reported in the human experience with hAAT), the association of T1D and type 1 hypersensitivities forms an obvious basis for future investigations, including development of protocols to avoid anaphylaxis and gain optimal protective effects in NOD mice or T1D patients, as well as understanding the immunological relationship between autoimmunity and the hypersensitivity reactions.

Acknowledgements

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